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Award Number:  DAMD17-03-1-0300

TITLE:  Gene Environment Interactions in Women with Breast Cancer and Secondary Lung Cancer

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CONTRACTING ORGANIZATION:  Georgetown University Medical Center
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REPORT DATE:  July 2004

TYPE OF REPORT:  Annual Summary

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

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# 20050121 004

# REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE July 2004 | 3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 2003 - 30 Jun 2004) |
|---|---|---|

**4. TITLE AND SUBTITLE**
Gene Environment Interactions in Women with Breast Cancer and Secondary Lung Cancer

**5. FUNDING NUMBERS**
DAMD17-03-1-0300

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract (Maximum 200 Words)** *(abstract should contain no proprietary or confidential information)*

Radiotherapy has become a standard treatment for breast cancer, however, few studies have examined individual susceptibilities to risks from radiation exposure. Lung cancer following breast cancer has been associated with radiation exposure and this increased lung cancer risk has been shown to be even higher with tobacco exposure. We are identifying p53 mutations in breast and lung tumors using the Affymetrix GeneChip system and examining the methylation status of five genes important in the progression of breast or lung cancer. We are also describing ER alpha and ER beta expression using immunohistochemistry. We have received 245 of the expected 300 tumors from cases and controls and all samples have been processed and DNA extracted. 160 samples have been analyzed for p53 mutations, 230 samples have been bisulfite modified for methylation assays, 200 methylation assays have been completed, and 145 breast tumors have been stained for ER alpha. This project may have significant clinical impact by providing additional information on risk levels to women choosing a breast cancer therapy. Additionally, this research may provide new data on the susceptibilities of women with multiple primary cancers and on hormone related gender differences in cancer risk.

**14. SUBJECT TERMS**
breast cancer, lung cancer, biomarkers, second cancers

**15. NUMBER OF PAGES**
14

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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## Introduction

Radiotherapy has become a standard treatment for breast cancer, however, few studies have examined individual susceptibilities to risks from radiation exposure. Lung cancer following breast cancer has been associated with radiation exposure and this increased lung cancer risk has been shown to be even higher with tobacco exposure. Identification of molecular markers of radiation exposure may allow the distinction of groups of women susceptible to secondary lung cancer or multiple cancers and of women more significantly affected by smoking. Additionally, identification of molecular markers in breast and lung tumor tissue may suggest a common etiology for breast cancer and secondary lung cancer. To study the risks associated with radiotherapy for breast cancer, we are using samples from the Swedish Cancer Registry, which contains information on approximately 95% of all cancer cases in Sweden.

We are looking at breast and lung tumor tissue for mutations in p53, which is involved in a radiation response pathway and is strongly associated with DNA damage from smoking. We are also comparing p53 mutations between women who did or did not receive radiotherapy and between smokers and nonsmokers with or without radiotherapy. Methylation of DNA is a key factor in the regulation of gene transcription and has been shown to contribute to carcinogenesis by blocking transcription of tumor suppressor genes. Based on this knowledge, we will be assessing the methylation of several genes known to be involved in cancer progression. Additionally, we are performing immunohistochemical assays to describe ER alpha expression in breast and lung tumors and ER beta expression in breast and lung tumors. This project may have significant clinical impact by providing additional information on risk levels to women choosing a breast cancer therapy. Additionally, this research may provide new data on the susceptibilities of women with multiple primary cancers and on hormone related gender differences in cancer risk

## Body

### Background

Radiation exposure is indicated as a risk factor for several cancers, but the study of radiation risks has been limited because of difficulties in measuring individual exposures and subsequent susceptibilities. Occupational studies have demonstrated that uranium and plutonium workers have an increased risk of lung cancer (1-3) as well as Japanese atomic bomb survivors, but individual exposures are difficult to precisely quantify. Studies of secondary cancer risk after radiotherapy are better able to establish radiation doses and link them to risk. Women with a history of breast cancer treated with radiotherapy in these studies had a secondary lung cancer risk that increased 2-3 fold for nonsmokers and 30 fold in smokers who receive radiation therapy (4,5). Overall, radiotherapy causes about 7-9 additional cases of lung cancer per 1000 women over a 10 year period (4,6). While the data is consistent, studies have been small and lacking in reliable information on radiation and smoking dose (7) and none have explored the effects of radiation and smoking in women with breast cancer at a molecular level.

## p53

The p53 gene is an appropriate subject for the study of cancer etiology, exposure, and susceptibility because of its many roles in cellular processes, including maintenance of genomic stability, apoptosis, DNA repair, and cell cycle control (8-12). p53 is upregulated in response to DNA damage by radiation (13-15), and cell lines with mutated p53 are hypersensitive to point mutations following radiation therapy (16). Examples of specific carcinogen exposures linked to cancers via p53 mutation mechanisms include ultraviolet (UV) light exposure and skin cancer (17-19) and dietary aflatoxin B1 exposure and liver cancer (20,21). The p53 tumor suppressor gene is the most commonly mutated gene in cancer; it is mutated in 40% of breast tumors (22,23) and 50% of lung tumors (24,25). p53 mutation frequency varies by tumor site and histological type (14), indicating that cancers occur through different pathways and due to different exposures at the cellular level. In breast cancer, studies indicate that the p53 mutation spectrum differs by race and geography (26,27) suggesting differences in etiology that might be environmental or genetic (28-30). In lung cancer, the type and frequency of p53 mutations have been correlated with smoking, and a study of uranium workers found a p53 mutational spectra in the workers' lung tumors that differed from the lung tumors of smokers (31). Studies in other populations, however, have not found differences in mutational spectra (54-58). Although there is conflicting data, significant evidence suggests that there may be a difference in the mutational spectra of women who develop secondary lung cancer with smoking and radiation in contrast to smoking alone (31,59).

## Estrogen Receptor

Steroid receptors are required for normal lung maturation and function. ER has a well-known role in the progression, treatment, and prognosis of breast cancer and may play a role in lung cancer as well. Gender differences in risk of lung cancer suggest that hormones and their receptors may influence the biology of lung cancer. Previous studies of ER expression and gender in lung cancer present varying reports, perhaps due to the lack of standardized techniques at the time (62-64, 69). A recent study found both ER alpha and ER beta in normal and tumor tissue in lung, with lower levels of both in the tumor samples (68). Studies conflict on whether ER has a prognostic value for lung carcinoma (51,60,61). However, there is significant evidence indicating an increased risk of adenocarcinoma of the lung with estrogen replacement therapy and an even higher risk in smokers who receive estrogen replacement therapy (53). These findings support the possibility that exogenous hormones play a role in the etiology of lung cancer in women. The significant implications of estrogen acting in the development of lung tumors and concerns about prior studies call for an examination of the presence of ER in women with breast and secondary lung tumors. There may be a shared hormonal etiology for these breast and secondary lung tumors and a potentially greater role for ER expression in the development of secondary lung cancer in nonsmoking, radiation treated women. Additionally, women with two primary cancers may represent a phenotype of increased estrogenicity or sensitivity.

## Methylation

Methylation of DNA is an epigenetic feature of DNA known to contribute to regulation of gene expression and to maintaining genome stability. DNA methyltransferases add methyl groups to the 5' cytosine residues of the dinucleotide CpG (32-34). Abnormal methyl patterns are consistently found in cancer, including hypermethylation of promoter regions and genome-wide and gene-specific hypomethylation (35). Both DNA hypermethlation and hypomethylation occur early in tumorigenesis and are thought to contribute to tumor progression, but whether abnormal DNA methylation is a consequence or a cause of cancer has not been established (36).

Several genes involved in breast and lung cancer are known to have abnormal methylation patterns. The DNA repair enzyme $O^6$-methylguanine-DNA methyltransferase has been shown to be frequently inactivated in lung cancer by aberrant promoter methylation (42,53). The BRCA1 gene is involved in maintenance of genomic integrity and studies have found it to be hypermethylated in 13%-29% of sporadic breast cancer (43,44). The tumor suppressor gene p16 is involved in cell cycle control and hypermethylation of its promoter has been shown to decrease p16 expression (45). 20%-40% of breast cancers have hypermethylated p16 (46,47). The estrogen receptor (ER) is downregulated in some breast cancers and lack of ER is associated with a poor prognosis. Hypermethylation of the ER promoter region has been detected in 63% of ER negative breast tumors (48) and has also been reported in lung tumors (49). E-cadherin is a transmembrane glycoprotein this is involved in cell adhesion. Aberrant CpG island methylation of E-cadherin has been found in breast cancer at a rate of 26-48% (70-72); in lung cancer, the rate is 18-33% (67). The retinoic acid receptor β (RAR-β) is a ligand activated transcription factor that is known to exert antiproliferative, differentiating, and apoptosis-induced effects on different types of tumors (73). In primary breast cancer specimens, 21-58% of specimens are hypermethylated at RAR-β (74-75) and in nonsmall cell lung cancer, 40% of specimens are hypermethylated RAR-β (67). Specific methylation patterns have been demonstrated to be associated with lung cancer and breast cancer and can provide valuable information on pathways in the development and progression of tumors.

## Research Accomplishments

**Task 1**: To determine the mutational spectra of the p53 tumor suppressor gene in paired, non-synchronous breast and secondary lung tumors in women. (Months 1-12)

  a. Extract DNA from slides of breast and lung tumor tissue from 160 case and control Swedish Cancer Registry samples.
  b. Sequence DNA extracted from samples using PCR amplification and the Affymetrix microarray system, including 20% repeated for quality control.
  c. Analysis of sequence data based on radiotherapy and smoking status.

The first task for this project is to extract DNA from breast and lung tumor tissue and to use extracted DNA in the Affymetrix microarray system to detect mutations in the p53 gene. We have received 245 of approximately 300 expected case and control tumors from our collaborators

b.  Perform immunohistochemical assays using Thyroid Transcription Factor-1 (TTF1) antibody on lung tumors from cases.
c.  Analysis of slide staining.

The second task for this project is to use immunohistochemistry to determine the ER alpha and beta status of the breast and lung tumors and to establish the primary tumor status of the lung samples.  5 micron slides obtained from the tumor blocks are stained for ER expression using ER alpha monoclonal antibody F-10 from Santa Cruz Biotechnology (Santa Cruz, CA), which recognizes the carboxy terminus of the receptor protein.  Citrate acid buffer is used for antigen retrieval, the antibody at 1:25 dilution for 1 hour at room temperature, followed by the StriAveGen Multilink Kit, staining with diaminobenzidine chromogen solution (DAB), and counterstaining with hematoxylin (all reagents from Biogenex; San Ramon, CA).  Slides are examined by microscope for the presence of ER staining and compared to the positive and negative control slides for each experiment.  Determination of positive or negative expression status is made using the Allred scoring system, where numerical scores from 0-5 for proportion of tumor stained and 0-3 for intensity of staining are added for a final score; two or higher is considered positive for ER expression (76).

Optimization studies were done to establish the correct dilution of the antibody for our protocol and to identify positive and negative control tissues.  135 breast tumor samples have been successfully stained for ER alpha and 18 samples have failed due to loss of tissue during staining process.  84 breast tumors have stained positive and 47 negative for ER alpha.  All slides will be double read and 20% will be repeated for quality control.

Evidence for the presence of ER alpha in lung tissue is conflicting, ranging from 0-80% in published studies (62-64, 68).  ER alpha staining was completed in 33 lung tumors, resulting in 3 positive and 30 negative samples.  We were concerned that the low rate of positive samples could be related to the possible presence of variant forms of ER alpha in lung tumor tissue.  If the monoclonal antibody we used in the lung stains was unable to detect an active variant form of ER alpha in the lung samples, we might have an inaccurate positive rate.  This led us to compare three antibodies, F-10 monoclonal from Santa Cruz Biotechnology, ID5 monoclonal from Dako, and a polyclonal from US Biological, for differences in staining in a series of lung tumor test samples.  This testing was unable to demonstrate differences in percent positive staining among the three antibodies.  With this information and our pathologist's opinion that the lung slides are correctly stained for ER alpha, we will continue to use the F-10 antibody to stain lung slides for ER alpha.  This data will be useful for establishing the primary status of the lung tumors when compared with data from the matching breast tumors.

Evidence for the presence of ER beta in lung tissue is stronger than for ER alpha (68-69).  Immunohistochemical staining for ER beta has been done using a polyclonal antibody from Upstate Biotech (Lake Placid, NY) directed at the A/B region of the receptor protein.  Optimization of the antibody was performed, control tissues for future studies were identified, and peptide inhibition assays using a recombinant ER beta protein were underway to validate the antibody.  However, through our collaboration with Swedish researchers, a lab with considerable experience with ER beta antibodies was identified and asked to perform the ER beta staining on all samples.  As ER beta immunohistochemistry is not yet a well established assay, especially in

lung tumors, this collaboration will ensure that our assays are performed in the best possible environment by knowledgeable technicians.

In order to confirm that the lung tumors from cases are primary lung cancer and not metastatic adenocarcinoma, we are performing immunohistochemical staining for thyroid transcription factor-1 (TTF1). Several reports have shown that this is an excellent marker for lung cancer (76% staining of adenocarcinomas) and does not stain breast cancer (77-82). We are using the 8G7G3/1 clone (83), a monoclonal antibody from Zymed Laboratories (San Francisco, CA). The TTF1 antibody was optimized for antigen retrieval and dilution of the antibody at 1:300 for staining at room temperature for 1 hour. The antibody was used on a set of 20 test lung adenocarcinoma samples and 75% of the samples were positive. 40 lung tumor samples were then stained, resulting in 5 positive and 35 negative. This low rate of positive staining, combined with an initial pathology review that could only concretely establish 47% of the lung tumors as primary (more information from medical records was requested before a final decision would be made), raised questions about the quality of the slides after transport from Sweden and the primary status of the lung tumors. As a result, the cases are being reviewed by another pathologist at the Karolinksa Institute in Stockholm and new slides are being cut from tumor blocks for immediate staining with TTF1 in Stockholm. Results will be discussed between pathologists and tumors for which an agreement cannot be reached will be excluded.

**Task 3**: To determine methylation status of *GSTP1, p16, BRCA1, ER, 06MGMT,* and *cyclinD2* in breast and secondary lung tumors in women. (Months 25-36)

   a. Perform PCR-based methylation assays on DNA extracted from 160 case and control Swedish Cancer Registry samples and identify DNA fragments using gel electrophoresis, including 20% quality control.
   b. Analysis of methylation patterns between breast and secondary lung tumors.

The third task for this project is to determine the methylation status of a panel of genes in breast and lung tumor tissue. Continued review of published and unpublished data has resulted in adjustments to the panel of genes, specifically, the removal of *GSTP1* and *cyclinD2* and the addition of *E-cadherin* and *RARbeta2*. *BRCA1* will be studied only in breast tumors and *O6MGMT* in lung tumors only. DNA extracted from tumor slides is subjected to bisulfite treatment, which results in the deamination of unmethylated cytosines. Deaminated cytosines become uracils, which are recognized as thymines by the Taq polymerase used in PCR. PCR is then performed using primers that differentiate between the methylated sequences and the unmethylated sequences, where thymines are substituted for cytosines.

Considerable effort was put into developing assays using methylation specific PCR and gel electrophoresis to detect methylation, but concerns about sensitivity and the amount of DNA used in the assay prompted the adoption by our lab of real-time PCR assays to detect promoter hypermethylation. We are currently using a Taqman assay, according to the method of Jeronimo et al. (84). Modified DNA (15 ng) is used as template with specific primers and probes corresponding to the methylated sequence. The primer and probe sequences for *p16* and *MGMT*

Taqman method have been published (85). Those for *E-cadherin* have been provided by Dr. Sidransky at Johns Hopkins University. Sequences for *BRCA1* and *ER* have been designed in our lab using ABI PrimerExpress software. Our lab is attempting to identify an assay to validate the presence of modified, amplifiable DNA in samples that are negative for methylation in all genes tested. Possibilities include *Beta-actin* and unmethylated *p16*.

200 samples have been bisulfite modified in preparation for the Taqman PCR assays. 60 breast tumors and 70 lung tumors have been tested for p16 methylation, resulting in 10% of breast tumors and 19% of lung tumors positive. 70 lung tumors have been tested for *O6MGMT*, resulting in 11% positive. Many samples have been tested in the *BRCA1, ER, E-cad*, and *RARbeta2* assays, but quality control tests have failed, so we are not reporting them at this point. These failures may be due to low quantity and low quality of modified template used in the assays, so we are exploring methods to improve the template. Whole genome amplification after bisulfate modification may improve performance of these samples in methylation assays while avoiding additional DNA extractions from limited tissues. All positive results will be confirmed with direct sequencing and 20% of samples repeated for quality control.

## Training

I have received additional training through attendance at the National Cancer Institute's course in Principles and Practice of Cancer Control and Prevention in July 2003, attendance at the September 2003 American Association of Cancer Researchers Molecular Epidemiology conference, and attendance at the Johns Hopkins University School of Public Health Graduate Institute for Epidemiology and Biostatistics in July 2004. I continue to be enrolled in the Tumor Biology Program Journal Club and Tumor Biology Data Meeting seminars and participate in bimonthly lab meetings and weekly meetings with Dr. Shields for students and postdoctoral fellows in the lab.

# Key Research Accomplishments

- Received, recorded, and labeled 245 samples
- Extracted DNA from 243 samples
- p53 mutation analysis by Affymetrix Gene Chip of 161 samples
- 135 breast tumors stained for estrogen receptor alpha
- 200 samples bisulfite modified
- 130 samples assayed for *p16* methylation
- 70 samples assayed for *O6MGMT* methylation

# Reportable Outcomes

-Abstract presented at Lombardi Cancer Center Research Fair 2004
    Georgetown University, Washington, DC
-Data presented at the Tumor Biology Program Data Meeting, September 2003
    Georgetown University, Washington, DC

## Conclusions

Though important questions have risen in the past year of work on this project, namely the verification of primary status of the case lung tumors and reproducibility of the methylation assay results, a significant amount of progress has been made toward completing the proposed assays. Use and evaluation of methylation assays has led to improvements to the assays used throughout the lab. Work with the TTF1 antibody and pathology reviews has indicated the need for a more thorough and careful review of primary lung tumors to establish them as non-metastatic, which could have implications for previous studies. The development of alternate methods for multiplex amplification of samples with low quality or low quantity DNA, identified by weak PCR results upon visualization by gel electrophoresis, has been useful for other studies in our lab and will have a large impact on the study of which this project is a subset.

There is significant evidence that indicates an increased risk for developing secondary lung cancer in women treated with radiotherapy for breast cancer and an additional risk for women who smoke. It is important to determine molecular markers for the susceptibility of breast cancer patients to lung cancer to improve information for treatment decisions. Markers in lung cancer may correlate with cancers that develop from radiotherapy alone, in combination with cigarette smoking, or with smoking alone. It is also important to examine the possibility that a particular predisposition, unrelated to any significant family history, has made these subjects more susceptible to different exposures, resulting in multiple primary tumors. It may be possible to define a unique subpopulation sensitive to hormonal risk factors for breast and/or lung cancer or to an alternate mechanism that could cause susceptibility to breast and lung cancer. We hypothesize that there will be differences in p53 mutation spectra, methylation status, and ER expression among women who are treated for breast cancer with radiotherapy and develop secondary lung cancer compared to women treated with radiotherapy who do not develop lung cancer, which may be useful in defining subpopulations sensitive to radiation therapy.

Women receiving radiation therapy following surgical treatment for breast cancer are believed to have improved disease free survival. For overall survival, however, benefits of radiotherapy have been more difficult to prove, especially for older women or those with a good prognosis. With information on increased risks based on radiation dose, breast cancer markers, history of smoking, and other factors, women can make more informed decisions about their breast cancer treatment. For women who elect to undergo radiotherapy, further screening and prevention methods may be devised. Women who smoke may be compelled by this additional risk information to quit smoking before radiotherapy. The degree of risk examined in this study is already considered to be at a level of importance similar to other risks of concern. This study will be significant because it is larger than previous studies and can provide new data about radiation carcinogenesis and interactions with smoking through the use of molecular markers.

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